Remarks

Oath/Declaration

The Examiner has indicated that the Declaration is defective on the grounds that (i) it does not identify the mailing address of each inventor and (ii) it does not identify the U.S. provisional application to which priority is claimed. Applicants submit that the information missing in the Declaration is not necessary for further consideration of the claims and therefore, in accordance with 37 C.F.R. 111(b), Applicants hereby requests that the requirement to correct the defects be held in abeyance until an indication of allowable subject matter is received.

Drawings

The drawings are objected to because Figure 1 appears handwritten and the pictures of the gels in Figures 3 and 4 have poor resolution. Amended drawings that address these concerns are enclosed herein.

Rejections under 35 U.S.C. § 112

Claim 61 stands rejected as failing to comply with the written description requirement. Claim 61 has been canceled, rendering the rejection moot.

Claims 27, 33, 103, 34, 35, 39, 45, 60, 41, 59, 61, 98-100, and 101 stand rejected as being indefinite on various grounds. These claims have been canceled, rendering the rejection moot.

Claim 32 stands rejected on the ground that the recitation of "proteins" lacks antecedent basis. Claim 32 has been amended to depend from claims 63, 84, and claims dependent therefrom. Applicants submit that proper antecedent basis for "proteins" is found in claims 63 and 84, from which claim 32 now depends. Withdrawal of the rejection is respectfully requested.

Claim 52 stands rejected on the ground that the recitation of components remaining in the modified sample" lacks antecedent basis. Claim 52 has been amended to depend from claims 63, 84, and 85. Applicants submit that proper antecedent basis for "the proteins in the modified sample" is found in claims 63 and 84, from which claim 52 now depends. Withdrawal of the rejection is respectfully requested.

Claims 105-107 stand rejected on the ground that the recitation of "one or more immunoglobulins" renders the scope of the Markush group unclear. These claims (and claim 104)

have been amended to recite "immunoglobulins" to indicate that each immunoglobulin is a member of the Markush group. Withdrawal of the rejection is respectfully requested.

Rejections under 35 U.S.C. § 102

Claims 27-42, 44-62, 98-105, and 108-109 stand rejected as being anticipated by Hutchens & Yip (U.S. Pat. No. 6,225,047), hereinafter "Hutchens & Yip". With the exception of claims 32, 52, 62, 104 and 105, all of these claims have been canceled. Claims 32, 52, 62, 104 and 105, as amended, are dependent on claims 63 and 84.

Claim 63, as amended, recites a method for separating proteins from a sample that contains proteins and recovering a modified sample for analysis of remaining proteins comprising: removing at least two specific predefined proteins from a sample ...and recovering the modified sample, wherein the removing step comprises contacting the sample with an affinity binding composition comprising: a first and second solid phase matrix contacting each other, wherein each solid phase matrix comprises a plurality of particles, and wherein the particles of the first and second solid phase matrices are present as a mixture in said affinity binding composition; a first receptor immobilized on said first solid phase matrix, capable of specific binding to a first protein but not a second protein; and a second receptor immobilized on said second solid phase matrix, capable of specific binding to the second protein but not the first protein.

Claim 84, as amended, recites a method for separating proteins from a sample that contains proteins and recovering a modified sample for analysis of remaining proteins comprising: removing at least two specific predefined proteins from a sample...and recovering the modified sample, wherein the removing step comprises contacting the sample with an affinity binding composition comprising: a plurality of solid phase matrices arranged such that each solid phase matrix is in contact with at least one other solid phase matrix; and a plurality of receptors having different protein binding specificities, wherein the receptors are immobilized on the plurality of solid phase matrices such that each solid phase matrix has a different protein binding specificity, wherein each solid phase matrix comprises a plurality of particles, and wherein the particles are present in the affinity binding composition as a mixture.

Hutchens & Yip do not teach or suggest the use of affinity binding compositions having the characteristics recited in claim 63 or claim 84. Hutchens & Yip teach (i) substrates that are strips, which may be attached to one another; and (ii) substrates that are plates having wells containing

populations of beads such that the bead(s) in any single well have the same adsorbent immobilized thereon. The first of these embodiments does not comprise particles as recited in each of the amended claims. The second of these embodiments does not teach affinity binding composition comprising solid phase matrices "contacting each other" or "in contact" with another solid phase matrix, wherein the solid phase matrices comprise a plurality of particles present in a mixture, as recited in claims 63 and 84. Withdrawal of the rejection is respectfully requested.

Claims 63-97 stand rejected as being anticipated by Mehta, et al, (U.S. Pat. No. 6,632,655), hereinafter "Mehta". A number of these claims have been canceled, therefore the following remarks concern claim 63 and other remaining claims, as amended. The Examiner states that Mehta describes a method comprising the steps of removing at least two specific predefined ligands and recovering a modified sample comprising a plurality of remaining components wherein the removing step comprises contacting the sample with an affinity binding composition comprising a first and second solid phase matrix contacting each other, an immobilized first receptor capable of specific binding to a first ligand but not a second ligand and an immobilized second receptor capable of specific binding to a second ligand but not the first ligand. The Examiner points to Mehta's mention of "subtractive hydridization" as involving removal of two specific predefined ligands. Claim 63 has been amended to recite that the ligands and components are proteins. In contrast, subtractive hybridization involves mRNA. Therefore Applicants submit that the cited portion of Mehta is no longer relevant. The rejection should be withdrawn for the foregoing reason alone.

Furthermore, Applicants respectfully submit that even if the terms "ligands" and "components" had been retained in the instant claims, Mehta does not teach the claimed invention for each of the following additional reasons.

Firstly, claim 63 as amended is drawn to an embodiment of the invention in which removal of the proteins is accomplished using an affinity binding composition comprising a *mixture* of particles, which is distinct from the particle sets taught by Mehta. The affinity binding composition recited in claim 63 comprises a mixture of first and second solid phase matrices contacting each other; a first receptor immobilized on said first solid phase matrix, capable of specific binding to a first protein but not a second protein; and a second receptor immobilized on said second solid phase matrix, capable of specific binding to the second protein but not the first protein, wherein each solid

phase matrix comprises a plurality of particles, and the particles are present in the affinity binding composition as a mixture. The amendment incorporates features of claims that have been canceled (e.g., claims 72 and 79). With respect to those claims, the Examiner states that Mehta describes a method in which sets of particles are "homogeneous" but does not explain how this renders the claims anticipated.

Applicants respectfully submit that the particle sets of Mehta are clearly distinct from those of claim 63. Mehta defines a "set" of particles as "a group or packet of particles having similar or identical constituents" (col. 7, lines 16-17). Mehta does not explain what is meant by "homogeneous". The ordinary meaning of the term is "of the same or similar nature or kind" (The American Heritage Dictionary, Second College Edition, Houghton Mifflin, Boston, 1985). The affinity binding composition of claim 63 comprises first and second solid phase matrices comprising particles that comprise *different receptors* and are present as a *mixture*. Applicants submit that since such an affinity binding composition contains a mixture of particles having *different constituents* it does not fall within Mehta's definition of a "homogeneous particle set".

Applicants note that Mehta also discusses "heterogeneous" sets of particles (col. 53, lines 6-24). The ordinary meaning of the term "heterogeneous" is "consisting of or involving dissimilar elements or parts" (The American Heritage Dictionary, Second College Edition, Houghton Mifflin, Boston, 1985). Mehta does not explain what is meant by "heterogeneous set', particularly in view of the general definition of "set". Mehta does not indicate in what respect the particle sets are "heterogeneous". They may be heterogeneous with respect to size, shape, or composition or in the sense that some or all of the particles may have multiple classes of "molecular tags" attached thereto. Regardless of the precise meaning ascribed to "heterogeneous", Mehta's "heterogeneous" set of particles must have "similar or identical constituents", in accordance with the definition of a "set" of particles, which contrasts with the mixtures of particles recited in the instant claims, which comprise different receptors on the particles of the solid phase matrices.

Mehta's description is so lacking in specificity that Applicants submit that an interpretation of either "heterogeneous" or "homogeneous" sets of particles as referring to an affinity binding composition comprising a mixture of first and second particulate solid phase matrices contacting each other; a first receptor immobilized on said first solid phase matrix, capable of specific binding to a first protein but not a second protein; and a second receptor immobilized on said second solid phase matrix, capable of specific binding to the second protein but not the first protein, is based

entirely on hindsight and would contradict Mehta's own requirement for a set of particles as "a group or packet of particles having similar or identical constituents".

Thus, this rejection should be withdrawn for each of the above two reasons alone.

In addition, Mehta does not teach removal of *specific, predefined* ligands from a sample using *specific, predefined* receptors, followed by recovery of a modified sample. As discussed during the interview and indicated by Mehta, subtractive hybridization is used to identify mRNAs that are differentially expressed between two or more samples. Subtractive hybridization involves forming a sample containing a first population of mRNA species in contact with a second population of mRNA species, wherein the first and second populations originate from different sources, and recovering a modified sample comprising mRNA species from the first population that do not hybridize with mRNA species from the second population. Applicants submit that the mRNA species that are removed from the sample to form the modified sample are not "specific, predefined ligands", and the mRNA species that hybridize to the mRNA species that are removed are not "specific, predefined receptors" within any reasonable meaning of the term. Subtractive hybridization does not require knowing the identity (e.g., in terms of sequence or hybridization properties) of any mRNA species in either sample with sufficient specificity to allow selection of a specific, predefined receptor (e.g., complementary mRNA) that would bind to it.

Applicants note that Mehta discusses use of "antibody arrays" to perform "protein hybridization". However, Mehta does not teach recovering a modified sample after contacting the sample with the antibodies because Mehta is only interested in detecting proteins that are retained on the array, e.g., in order to compare expression profiles in different samples.

Furthermore, there is no teaching in Mehta that any of the particle sets would have antibodies attached thereto in amounts sufficient to remove 50% by weight of all protein in a sample, as recited in dependent claim 32.

Claim 84 as amended recites a method for separating proteins from a sample that contains proteins and recovering a modified sample for analysis of remaining components, wherein the removing step comprises contacting the sample with an affinity binding composition comprising: a plurality of solid phase matrices arranged such that each solid phase matrix is in contact with at least one other solid phase matrix; and a plurality of receptors having different protein binding specificities, wherein the receptors are immobilized on the plurality of solid phase matrices such that each solid phase matrix has a different protein binding specificity, wherein each solid phase

matrix comprises a plurality of particles, and wherein the particles are present in the affinity binding composition as a mixture. The amendment incorporates features from canceled claims, e.g., claim 87, and is supported by those claims. Applicants submit that Mehta does not teach the claimed method for reasons similar to those recited above with respect to claim 63. Mehta simply does not teach an affinity binding composition comprising a mixture of a plurality of particulate solid phase matrices such that each solid phase matrix has a different protein binding specificity, and certainly does not teach use of such an affinity binding composition for removing at least two specific, predefined proteins from a sample.

For each of the foregoing reasons, withdrawal of the rejection of claims 63, 84, and claims dependent therefrom is respectfully requested.

Rejections under 35 U.S.C. § 103(a)

Claims 106 and 107 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over Mehta in view of U.S. Patent No. 5,137,808 to Ullman, hereinafter "Ullman". The Office Action states that since Ullman teaches the use of affinity binding compositions for isolating and characterizing blood plasma components including a number of the proteins recited in claims 104-107, it would have been obvious for a person of ordinary skill in the art to modify the method for separating ligands, as taught by Mehta, with the use of compositions specific for blood plasma components because Ullman discovered a convenient, on-site means for testing a variety of analytes.

As set forth in MPEP §706.02(j), Contents of a 35 U.S.C. §103 Rejection, "To establish a *prima facie* case of obviousness, three basic criteria must be met. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations." See *In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991). Applicants respectfully submit that a *prima facie* case of obviousness has not been established, for each of the following reasons.

Applicants first note that Ullman does not teach the use of affinity binding compositions for isolating and characterizing blood plasma components but simply for detecting their presence and, optionally, quantifying them. As discussed during the interview, and acknowledged by the

Examiner, the fact that Ullman teaches the desirability of detecting blood plasma components does not provide motivation for the removal of such components from a sample. Furthermore, the fact that Ullman teaches the desirability of detecting blood plasma components does not provide motivation for recovering a modified sample from which the components have been removed, as recited in the instant claims. There would simply be no reason for recovery of the modified sample. For this reason alone this rejection should be withdrawn.

In addition, even if motivation to combine existed, which it does not, the combination of Mehta and Ullman still would not teach each of the features of the claimed invention since, as discussed above with reference to claim 63, (i) Mehta does not teach removing at least two specific predefined proteins from a sample, wherein the at least two specific predefined proteins are removed by binding to specific predefined receptors, each of which binds to a specific predefined protein; and (ii) the affinity binding composition recited in claims 63 and 84 is clearly distinct from the particle sets of Mehta. Mehta also does not teach removal of at least 50% by weight of all proteins in a sample, as in claim 32. Furthermore, Mehta certainly does not teach recovery of a modified sample comprising a plurality of proteins, following removal of at least two specific, predefined proteins.

In summary, as discussed during the Interview and acknowledged by the Examiner, there is no motivation to combine the teachings of Mehta and Ullman. Additionally, even if such a motivation existed, the resulting combination still would not teach each of the features of the claimed invention. Applicants therefore submit that the instant claims are not obvious for each of the foregoing reasons. Withdrawal of the rejection is respectfully requested.

Additional Claim Amendments

Claims 104-107 have been amended to recite an additional abundant protein, namely alpha 2 HS glycoprotein. Support is found in Table 1, on p. 25.

In conclusion, in view of the amendments and remarks presented herein, none of the cited art anticipates any of the claims pending in the instant application nor renders them obvious, and the application complies with the requirements of 35 U.S.C. §112. Applicants therefore respectfully submit that the present case is in condition for allowance. A Notice to that effect is respectfully requested.

If, at any time, it appears that a phone discussion would be helpful, the undersigned would greatly appreciate the opportunity to discuss such issues at the Examiner's convenience. The undersigned can be contacted at (617) 248-5000 or (617) 248-5071 (direct dial).

Please charge any fees associated with this filing, or apply any credits, to Deposit Account No. 50-1078.

Respectfully submitted,

Monica R. Gerber, M.D., Ph.D.
Registration Number 46,724

Date: July 8, 2005

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3954206_1.DOC

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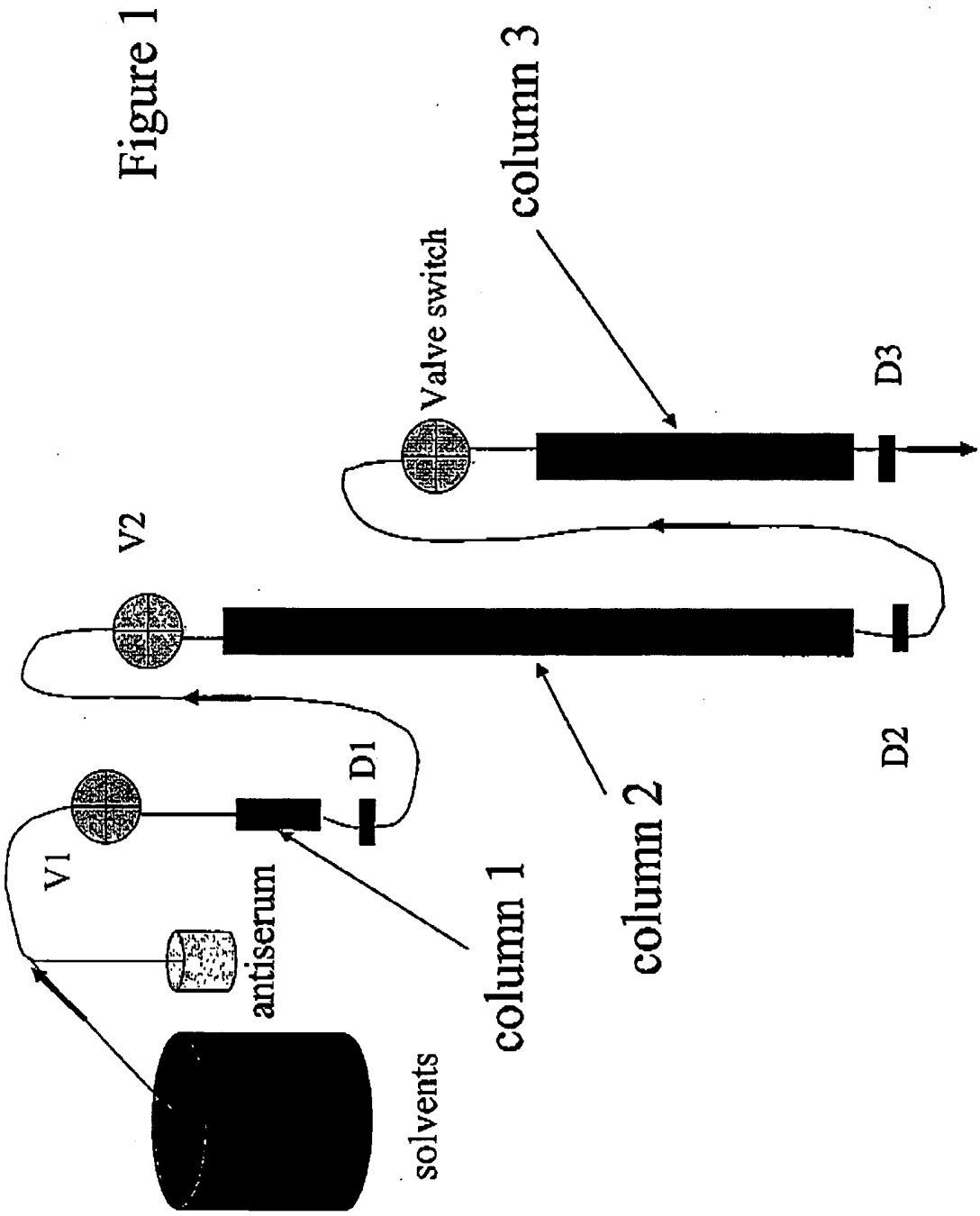
Two International Place
Boston, MA 02110
Phone, fax & email will not change.

Atty. Docket No.: 10030634-2
CHS No.: 2003309-0061

Figure 1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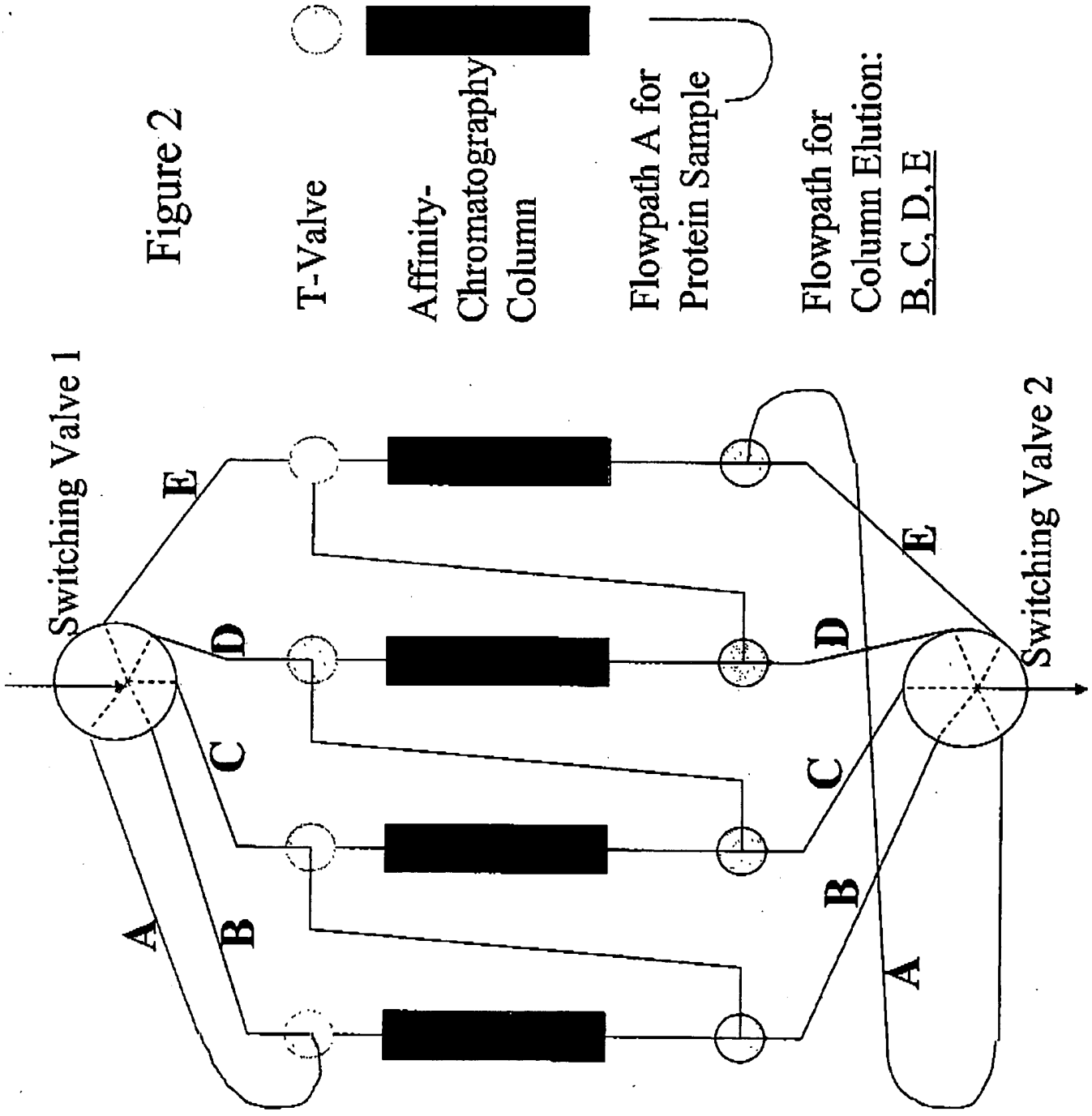
Valve switch
D3
V2
D2
column 2
D1
V1
column 1
antiserum
solvents

Figure 2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Affinity-Chromatography Column
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Flowpath for Column Elution: B, C, D, E
Switching Valve 1
Switching Valve 2
A
B
C
D
E

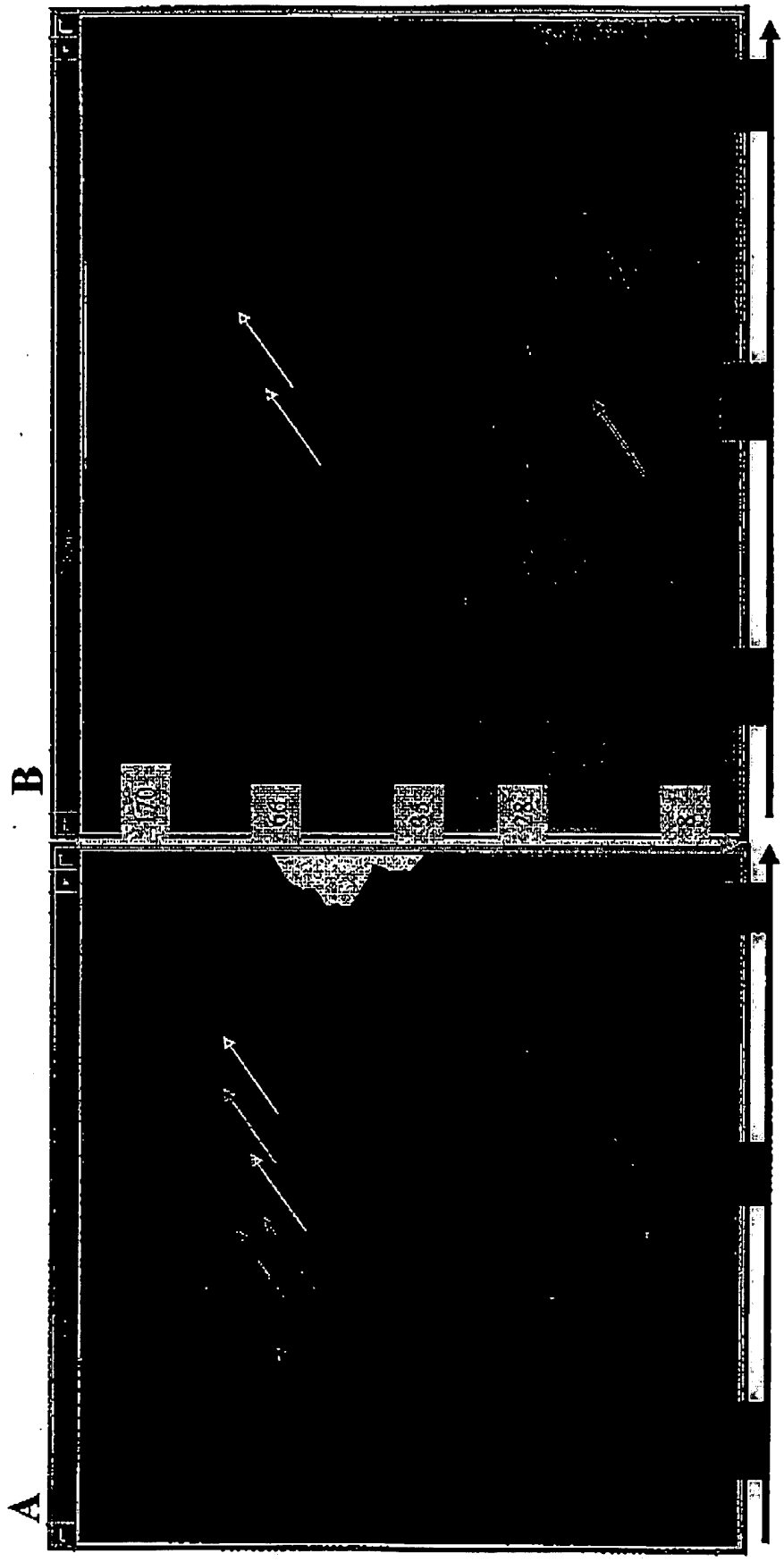

Figure 3

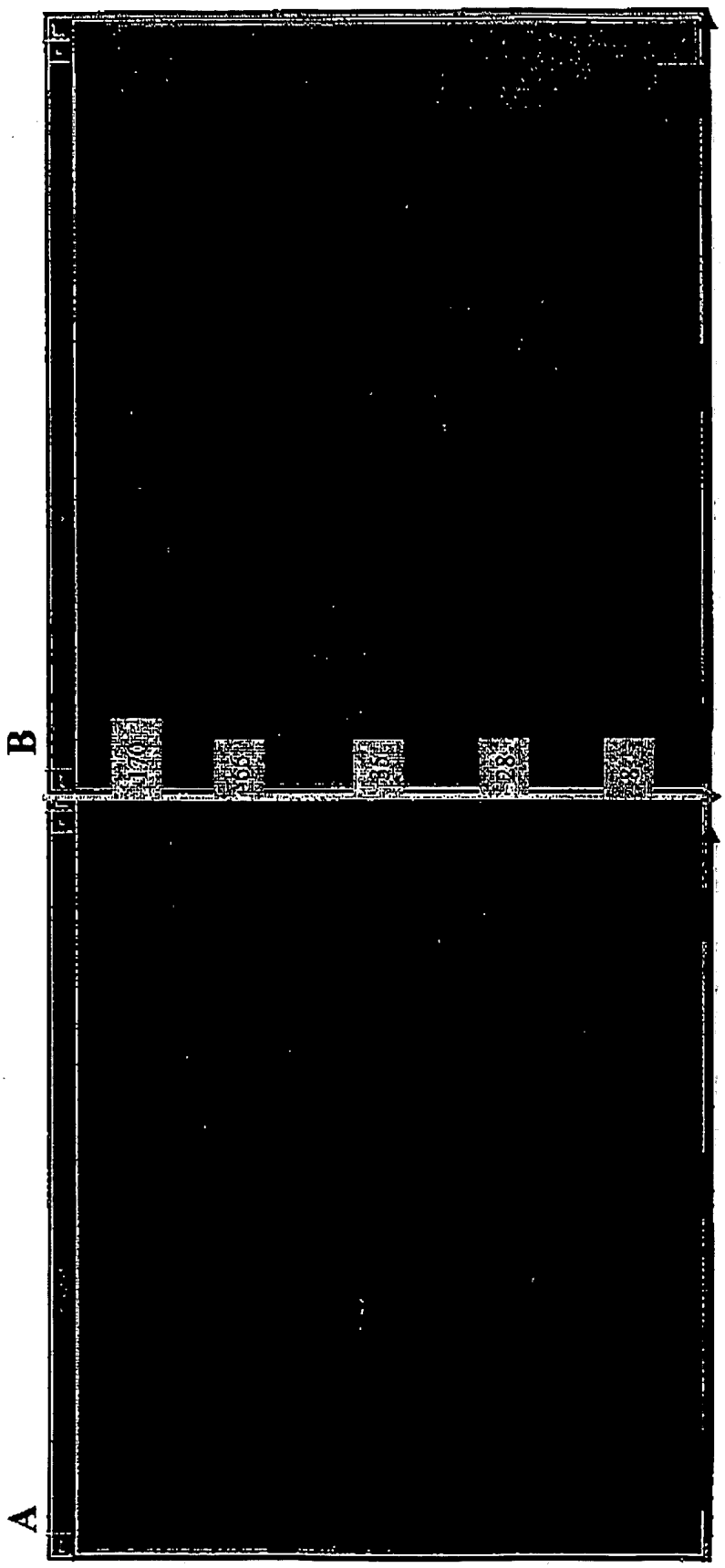

Figure 4

ATTORNEY DOCKET NO.: 10030634-2 (CHS No.: 2003309-0061)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Pieper, et al	Examiner:	Venci, D.J.
Serial No.:	09/977,358	Art Unit:	1641
Filing Date:	October 16, 2001		
Title:	IMMUNOSUBTRACTION METHOD FOR SAMPLE PREPARATION FOR 2-DGE		

BY FACSIMILE TRANSMISSION
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

STATEMENT

Pursuant to the duty of disclosure under 37 C.F.R. §§1.56, 1.97 and 1.98, Applicant requests consideration of this Information Disclosure Statement.

Type of Statement

The present Information Disclosure Statement is:

[ ] An *original* Information Disclosure Statement; or

[X] A *supplemental* Information Disclosure Statement.

Compliance with 37 CFR § 1.97

The present Information Disclosure Statement is being filed:

[X] Pursuant to 37 CFR § 1.97(b); no fee or certification is required:

[ ] Within three months of the filing date of a national application other than a continued prosecution application under § **1.53(d)**;

[ ] Within three months of the date of entry of the national stage as set forth in **§ 1.491** in an international application;

[ ] Before the mailing of a first Office action on the merits; or

[X] Before the mailing of a first Office action after the filing of a request for continued examination under § **1.114**.

[ ] Pursuant to 37 CFR § 1.97(c) after the dates listed above but before the mailing date of any of a final action under § **1.113**, a notice of allowance under § **1.311**, or an action that otherwise closes prosecution in the application; Applicant hereby *either*:

[ ] Certifies that *either*:

[ ] each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement; or

[ ] That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the

knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in § 1.56(c) more than three months prior to the filing of the information disclosure statement.; or

[ ] Includes herewith the fee set forth in § 1.17(p). Please charge Deposit Account No. 50-1078.

[ ] Pursuant to 37 CFR § 1.97(d), after the mailing date of any final action under § 1.113, a notice of allowance under § 1.311, or an action that otherwise closes prosecution in the application; Applicant hereby *both*:

[ ] Certifies that *either*:

[ ] each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement; or

[ ] That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in § 1.56(c) more than three months prior to the filing of the information disclosure statement.; and

[ ] Includes herewith the fee set forth in § 1.17(p).

Content of the Information Disclosure Statement

Applicant hereby makes of record in the above-identified application the reference(s) listed on the attached form PTO-1449 (modified). The order of presentation of the references should not be construed as an indication of the importance of the references.

Applicant includes copies of references as indicated below:

[X] A copy of each cited reference not indicated with an asterisk is included;

[ ] Copies of references indicated with an asterisk on the attached form PTO-1449 are not included pursuant to 37 CFR § 1.98(d) because they were previously provided to the United States Patent Office in an Information Disclosure Statement that complies with 37 CFR § 1.98(a)-(c) and was submitted in the following patent application that is relied upon in the present case for an earlier effective filing date under 35 USC § 120:

Serial Number	Filing Date	Status

[ ] Copies of English translations of one or more non-English references are included.

Applicant hereby makes the following additional information of record in the above-identified application:

Applicant certifies that the Information Disclosure Statement *either*:

[X] Does not contain non-English language citations;

[ ] Does contain non-English language citations, of which the following is a concise

explanation:

[ ] Includes one or more translations of a non-English citation.

Remarks

The submission of this Information Disclosure Statement should not be construed as a representation that a search has been made.

The submission of this Information Disclosure Statement shall not be construed to be an admission that the information cited in the statement is, or is considered to be, material to patentability as defined in § 1.56(b) .

The submission of this Information Disclosure Statement shall not be construed as a representation that the information cited in the Statement is, or is considered to be, in fact, prior art as defined by 35 U.S.C. §102.

It is respectfully requested that:

1. The Examiner consider completely the cited information, along with any other information, in reaching a determination concerning the patentability of the present claims;

2. The enclosed form PTO-1449 be signed by the Examiner to evidence that the cited patent(s) and publication(s) has (have) been fully considered by the Patent and Trademark Office during the examination of this application; and

3. The citations for the patent(s) and publication(s) be printed on any patent which issues from this application.

Notwithstanding any statements by Applicants, the Examiner is urged to form his or her own conclusions regarding the relevance of the cited reference(s).

Respectfully submitted,

Dated: July 8, 2005

Monica R. Gerber, M.D., Ph.D.
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Form PTO-1449 (REV. 8-83) U.S. Department of Commerce Patent and Trademark Office	Atty. Docket: 2003309-0061 10030634-2	In re Application No. 09/977,358
INFORMATION DISCLOSURE STATEMENT *(Use several sheets if necessary)*	Applicant: Pieper, et al.	
	Filing Date: October 16, 2001	Group: 1641

U.S. PATENT DOCUMENTS

Examiner's Initials	U.S. Patent No.	Applicant	Issue Date	Class	Subclass

U.S. PATENT APPLICATIONS

Examiner's Initials:	Application Number:	Applicant:	Filing Date:	Group:	Art Unit:
	2004/0072251	Anderson	April 15, 2004		
	2003/0032017	Anderson et al.	February 13, 2003		
	10/413,393	Bente, H. Bryan	April 15, 2003		

FOREIGN PATENT DOCUMENTS

Examiner's Initials	Document No.	Country	Date	Translation	
				Yes	No

OTHER DOCUMENTS

Examiner's Initials	Citation (Including Author, Title, Date, Pertinent Pages, Etc.)
	Wu, et al., "Targeted Proteomics of Low-Level Proteins in Human Plasma by LC/MS^n: Using Human Growth Hormone as a Model System", *Journal of :Proteome Research*, 1: 459-465, 2002.

EXAMINER	DATE CONSIDERED

EXAMINER: Initial if citation considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

3946941v1

UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICATION NUMBER	FILING DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	DRAWINGS	TOT CLAIMS	IND CLAIMS
10/413,393	04/15/2003	1743	834	10021053-1	2	20	4

CONFIRMATION NO. 3145

AGILENT TECHNOLOGIES, INC.
Legal Department, DL429
Intellectual Property Administration
P.O. Box 7599
Loveland, CO 80537-0599

FILING RECEIPT

OC0000000010203408

Date Mailed: 06/06/2003

Receipt is acknowledged of this regular Patent Application. It will be considered in its order and you will be notified as to the results of the examination. Be sure to provide the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION when inquiring about this application. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please write to the Office of Initial Patent Examination's Filing Receipt Corrections, facsimile number 703-746-9195. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections (if appropriate).**

Applicant(s)

H. Bryan Bente, Landenberg, PA;

Domestic Priority data as claimed by applicant

Foreign Applications

If Required, Foreign Filing License Granted: 06/05/2003

Projected Publication Date: 10/21/2004

Non-Publication Request: No

Early Publication Request: No

Title

Method for detecting a low abundance protein in a test sample

Preliminary Class

436

LICENSE FOR FOREIGN FILING UNDER
Title 35, United States Code, Section 184
Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

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The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Office of Export Administration, Department of Commerce (15 CFR 370.10 (j)); the Office of Foreign Assets Control, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

METHOD FOR DETECTING A LOW ABUNDANCE PROTEIN IN A TEST SAMPLE

TECHNICAL FIELD

The present invention relates generally to methods of protein analysis and, in particular, to methods for detecting low abundance proteins in a test sample.

BACKGROUND OF THE INVENTION

Protein analysis is widely used in diagnostic, medical, environmental, and industrial applications. Various analytical methods have been developed to separate and identify the proteins of interest in a test sample. Commonly used methods include gel electrophoresis, chromatography, mass spectroscopy, ELISA, immunoprecipitation, and Western blot. Generally, the proteins of interest are separated from other proteins in the test sample based on their physical properties (e.g., molecular weight, size, charge, and hydrophobicity) and/or immunological characteristics (e.g., immune-specific binding to antibodies). One common problem, however, is that the proteins of interest are often present at low levels in the test sample. The separation and identification of these low abundance proteins (LAPs) may be seriously affected by the background interference from the high abundance proteins (HAPs) in the test sample. For example, albumin and immunoglobulins are quite prevalent in serum samples and often interfere with the analysis of other serum proteins such as growth hormone or lymphokines. In order to enhance the accuracy of protein analysis of LAPs, it is desirable to diminish or completely remove the contribution of the background interference from the HAPs in the test sample.

One strategy to avoid HAP interference is to selectively remove the HAPs from the test sample using either non-specific means such as salt-precipitation, or specific means such as immuno-absorption. A major problem, however, is that many LAPs are associated with the HAPs through non-covalent interactions, such as ionic interactions, van der Waals interactions, hydrogen bonds, and hydrophobic interactions. Because these LAPs are physically bound to the HAPs, removal of the HAPs would also lead to the removal of the LAPs. The depletion of HAP-bound LAPs affects the accuracy of the protein analysis of LAPs in the test sample.

SUMMARY OF THE INVENTION

The present invention discloses a novel procedure of removing HAPs from a test sample without the concurrent removal of HAP-bound LAPs. The procedure therefore

allows the accurate detection of LAPs without the interference from the HAPs in the test sample. Specifically, the present invention provides methods for detecting LAPs in a test sample by treating the test sample with a proteolytic agent to release HAP-bound LAPs from the HAPs by fragmenting both HAPs and LAPs, removing the HAP fragments from the test sample, analyzing the LAP fragments, and identifying the LAPs in the test sample based on the characteristics of the LAP fragments. The present invention is most useful for the identification of LAPs that are bound to the HAPs in the test sample and are otherwise hard to separate from the HAPs.

In one embodiment, the present invention provides a method for detecting LAPs by treating the test sample with a proteolytic agent to fragment proteins. Typically, both the HAPs and LAPs in the test sample are degraded by the proteolytic agent. The degradation process disrupts the protein-protein interaction between LAP fragments and HAP fragments, and results in the release of the LAP fragments from the HAP fragments. The HAP fragments are removed from the sample. The unbound, LAP fragments are analyzed without the interference from the HAP fragments. The LAPs in the test sample are then identified based on the characteristics of the LAP fragments.

In another embodiment, the present invention provides a method for detecting LAPs by first isolating intact HAPs from the test sample. The HAPs, together with the LAPs that are bound to them, are then fragmented with a proteolytic agent. The fragmentation process generates both HAP and LAP fragments, disrupts protein-protein interactions between the HAP fragments and the LAP fragments, and releases the LAP fragments from the HAP fragments. The HAP fragments are removed from the reaction mixture and the unbound, LAP fragments are then analyzed without the interference of the HAPs. The LAPs that were bound to the isolated HAPs are identified based on the characteristics of the LAP fragments. In this embodiment, LAPs that do not bind to the HAPs are left in the test sample and are identified after the removal of HAPs using traditional methods.

Another aspect of the present invention pertains to a protein assay kit containing a proteolytic agent to fragment proteins for the disruption of associations between the LAPs and HAPs in a test sample, and a binding material or materials that bind specifically to the HAP fragments generated by the proteolysis of the HAPs with the proteolytic agent. Preferably, the binding material(s) is attached to a solid supporting material to facilitate the separation of the binding material(s) and the HAP fragments bound to them from other peptides in the test sample.

Other aspects of the invention will become apparent to the skilled artisan by the following description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention of this application is better understood in conjunction with the following drawings, in which:

Figure 1 is a flow chart describing an embodiment of the protein assay method of the present invention.

Figure 2 is a flow chart describing another embodiment of the protein assay method of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

The following detailed description is presented to enable any person skilled in the art to make and use the invention. For purposes of explanation, specific nomenclature is set forth to provide a thorough understanding of the present invention. However, it will be apparent to one skilled in the art that these specific details are not required to practice the invention. Descriptions of specific applications are provided only as representative examples. Various modifications to the preferred embodiments will be readily apparent to one skilled in the art, and the general principles defined herein may be applied to other embodiments and applications without departing from the scope of the invention. The present invention is not intended to be limited to the embodiments shown, but is to be accorded the widest possible scope consistent with the principles and features disclosed herein.

The present invention is generally directed to methods of protein analysis and, in particular, to the identification and/or quantification of LAPs in a test sample. The present invention overcomes the problem of background interference from HAPs by digesting both LAPs and HAPs with a proteolytic agent, and removing the resulting HAP fragments prior to the analysis of the LAP fragments.

With reference now to FIGURES 1 and 2, various embodiments of the protein assay method of the present invention will be described. As will be described in more detail below, the protein assay method may be used for the identification and/or quantification of any protein of interest in a test sample.

FIGURE 1 shows one embodiment of the protein assay method of the present invention, which is generally designated by the reference number 100. The first step in the method 100 is to add a proteolytic agent to a test sample (step 101). The test sample is then incubated under conditions that allow the degradation of proteins in the test

sample (step 103). Typically, both HAPs and LAPs are fragmented by the proteolytic agent in step 103. The fragmentation process disrupts the protein-protein interaction between HAPs and LAPs and releases the LAPs (most likely LAP fragments after the proteolysis) from the HAPs that they associated with before the proteolysis. The HAP fragments (and any undigested HAPs) are then removed from the test sample (step 105) and the LAP fragments are analyzed without the interference from HAPs (step 107). The LAPs in the test sample are then identified (step 109) based on the characteristics of the LAP fragments analyzed in step 107.

The test sample includes, but is not limited to, biological, physiological, industrial, environmental, and other types of samples. Of particular interest are biological fluids such as serum, plasma, urine, cerebrospinal fluid, saliva, milk, broth, cell lysates, and other culture media and supernatants, as well as fractions of any of them. The test sample may also be a particular fraction of one of these samples with fractionation accomplished by one or more known methods including, but not limited to, filtration, chromatography, electrophoretic methods, or affinity methods. Physiological fluids of interest include infusion solutions, buffers, preservative or antimicrobial solutions and the like. Industrial liquids include fermentation media and other processing liquids used, for example, in the manufacture of pharmaceuticals, dairy products and malt beverages. Other sources of sample fluid which are tested by conventional methods are contemplated as within the meaning of this term as used and can, likewise, be assayed in accordance with the invention.

A protein is considered a high abundance protein (HAP), if it constitutes more than 1% by weight of total protein in a test sample. The HAPs may also be defined arbitrarily relative to a low abundance protein (LAP) or proteins. For example, in a test sample containing multiple proteins, a protein may be present in an amount that is significantly greater than the amount of another protein in the same sample. Generally, if a first protein is present in an amount that is at least three-fold of a second protein in the same sample, the first protein may be considered an HAP relative to the second protein, while the second protein may be considered an LAP relative to the first protein. In this scenario, it is possible that, in some cases, the first protein (the HAP) may amount to less than 1% of the total protein in the sample. For example, protein A amounts to 0.5% of the total protein and protein B amounts to 0.1% of the total protein in a sample. Protein A may be considered an HAP relative to protein B, althought protein A constitutes less than 1% of the total protein in the sample. It is also possible that, in some other cases, the

second protein (the LAP) may amount to more than 1% of the total protein in the sample. For example, protein A amounts to 10% of the total protein and protein B amounts to 2% of the total protein in a sample. Protein B may be considered an LAP relative to protein A, althought protein B constitutes more than 1% of the total protein in the sample.

The proteolytic agent can be any agent that is capable of digesting the HAPs and releasing the HAP-bound LAPs from the HAPs. The proteolytic agent may be a protease, such as trypsin, or a mixture of proteases that hydrolyze a peptide bond between a pair of amino acids located in a polypeptide chain. The proteolytic agent may also be a chemical agent, such as cyanogen bromide (CNBr), which cleaves a peptide only on methionine residues, or a mixture of chemical agents. Many references list proteolytic agents and describe their use (for example, see Current Protocols in Protein Science, John E. Coligan, Ben M. Dunn, David W. Speicher, Paul T. Wingfield, eds. John Wiley & Sons, Inc. 1995-2001).

It should be noted that a typical test sample may contain hundreds of proteins and each protein may generate 10 to 50 fragments depending on the size of the protein and the proteolytic agent. A majority of the fragments are derived from the HAPs in the test sample. Therefore, it is necessary to remove the HAP fragments (step 105) before the characterization of the protein fragments derived from the LAPs. The removal need not to be 100%, but only to an extent that the remaining HAP fragments do not interfere with the characterization of the LAP fragments in the test sample. Accordingly, the completeness of the HAP removal in step 105 will be tailored to suit the characteristics of the particular assay system to be employed in step 107.

The removal of the HAP fragments (and any undigested HAP proteins) may be accomplished using conventional protein/peptide separation methods, which include, but are not limited to, chemical methods such as salt precipitation, physical methods such as chromatography, dialysis and filtration, and immunological methods such as affinity column and immunoprecipitation. Preferably, the HAP fragments and undigested HAPs are removed by methods based on HAP-specific immune-absorption. For example, anti-HAP antibodies may be attached to a solid supporting material, incubated with the digested test sample to allow binding of the HAP fragments to the antibodies, and removed from the test sample with the bound HAP fragments. The solid supporting material can take on a multitude of forms, such as beads, membrane, or the interior surface of a tube, vessel, or container. The solid supporting material can be mono- or multi-phasic, comprising one or more appropriate materials or mediums of similar or

different absorptive or other physical characteristics. The solid supporting material can be hydrophobic or hydrophilic, bibulous or nonporous. In its most efficient embodiment, the solid supporting material is carefully tailored to suit the characteristics of the particular assay system to be employed in step 107. Methods for coating a solid supporting material with antibodies are well known in the art.

Preferably, the anti-HAP antibodies are polyclonal antibodies or a mixture of monoclonal antibodies directed to different HAPs and different fragments of the same HAP, so that the antibody-coated beads or other surfaces are capable of capturing a majority of HAP fragments and remove them from the test sample in step 105. Because different proteolytic agents result in different protein fragments, different anti-HAP fragment antibodies will be required for each proteolytic agent used.

The LAP fragments in the test sample are then analyzed in step 107. The removal of the HAP fragments and undigested HAPs in step 105 should significantly reduce the HAP interference during the sample analysis. Typically, the LAP fragments in the digested test sample are separated and characterized using conventional peptide assay methods, which include, but are not limited to, chromatography, high performance liquid chromatography, mass spectrometry, and Edman degradation. A preferred method is mass spectrometry. Again, many references exist on these techniques. Examples are High Resolution Separation and Analysis of Biological Macromolecules, Part A Fundamentals, Barry L. Karger and William S. Hancock, eds. in Methods in Enzymology, Vol. 270, Academic Press, San Diego, CA, 1996; High Resolution Separation and Analysis of Biological Macromolecules, Part B Applications, Barry L. Karger and William S. Hancock, eds. in Methods in Enzymology, Vol. 271, Academic Press, San Diego, CA, 1996; Mass Spectrometry of Proteins and Peptides, John R. Chapman, Ed., Humana Press, Totowa, N.J., 2000; and Current Protocols in Protein Science, John E. Coligan, Ben M. Dunn, David W. Speicher, Paul T. Wingfield, eds. John Wiley & Sons, Inc. 1995-2001.

Depending on the assay method, analysis of the LAP fragments in step 107 can be qualitative and/or quantitative. After the analysis of the LAP fragments, the identity of the LAPs in the test sample is determined based on the characteristics of the LAP fragments. For example, if the amino acid sequence of an LAP fragment has been determined in step 107, the corresponding LAP can be identified by performing a search in protein databases such as GenBank and Swiss-Prot. Similarly, protein identification may also be based on other characteristics of an LAP fragment, such as the size, electrical

charge, secondary structure, and hydrophobicity of the LAP fragment. It is conceivable that a customized protein database may be constructed for the identification of LAPs using peptide characteristics determined in step 107 of the assay method 100. If the LAP fragments are quantified in step 107, it is also possible to quantify the corresponding LAPs since the amount of an LAP is proportional to the amount of the LAP fragments derived from the LAP.

FIGURE 2 shows another protein assay method of the present invention, which is generally designated by the reference number 200. In the method 200, the HAPs, together with any bound LAPs, are isolated from the test sample (step 201). A proteolytic agent is added to the isolated HAPs to form a reaction mixture (step 203). The reaction mixture is incubated under conditions that allow the fragmentation of the isolated HAPs and the HAP-bound LAPs (step 205). During the fragmentation process, the proteolytic agent degrades both the HAPs and the HAP-bound LAPs, and releases the LAPs fragments from the HAP fragments. In the next step, the HAP fragments are removed from the reaction mixture (step 207). The LAP fragments in the reaction mixture are then analyzed (step 209) and the identity of the HAP-bound LAPs are determined based on the characteristics of the LAP fragments (step 211). In a parallel step, the HAP-depleted test sample is also analyzed for the LAPs that do not bind to the HAPs (step 213).

In step 201, the undigested HAPs are preferably separated from the other proteins in the test sample using an immune-absorption based method, such as affinity chromatography or immunoprecipitation. Once separation has occurred, the HAPs are released from the immune-absorption material. Similar to assay method 100, the proteolytic agent in step 203 is chosen based on the characteristics of the isolated HAPs, and the incubation conditions in step 205 are determined based on the particular proteolytic agent used in the reaction. Optimal reaction conditions for each protease or chemical proteolytic agent are well-known in the art.

The HAP fragments can be removed from the reaction mixture using conventional chemical, physical and immunological protein separation methods. Preferred separation methods include affinity chromatography, immunoprecipitation, and immune-absorption on a membrane or a solid surface. After the removal of the HAP fragments, the LAP fragments in the reaction mixture can be analyzed and the corresponding LAPs can be identified as described in assay method 100.

It should be noted that not all LAPs in the test sample are bound to the HAPs and co-isolated with the HAPs. Therefore, some LAPs are left in the test sample after the

removal of the HAPs in step 201. Accordingly, the HAP-depleted test sample is also analyzed for non-HAP-bound LAPs in step 213. This analysis may involve the fragmentation of the non-HAP-bound LAPs by a proteolytic agent, the characterization of LAP fragments, and the identification of the LAPs based on the characteristics of the LAP fragments.

As understood by one skilled in the art, the methods of the present invention are most useful in detecting LAPs or LAP profiles in test samples when the identity of the LAPs are unknown before the analysis. However, it is also conceivable that the methods of the present invention can be used to quantify a known LAP or LAPs without the interference from the HAPs in the same test sample. Specifically, the methods of the present invention may be used to more accurately quantify a known LAP that binds to HAPs in a test sample. In this scenario, the LAP(s) of interest can be released from the HAPs by proteolysis, the amount of one of the free, unbound LAP fragments may be determined by an immune-absorption based method such as ELISA or RIA. The amount of the LAP of interest can then be inferred from the amount of the proteolytic fragment derived from the LAP of interest. Preferably, the HAP fragments resulting from the proteolysis are removed prior to the quantification of LAP fragments to reduce interference from the HAP fragments.

Another aspect of the present invention pertains to a detection kit for LAPs. The protein assay kit contains a proteolytic agent to fragment proteins in a test sample for the disruption of associations between the LAPs and HAPs in the test sample, and a binding material or materials that bind specifically to the HAP fragments generated by the proteolysis of the HAPs with the proteolytic agent. Preferably, the binding material(s) is attached to a solid supporting material to facilitate the separation of the binding material(s) and the peptides bound to them from other proteins/peptides in the test sample.

The preferred embodiments of novel methods for analyzing LAPs in a test sample are intended to be illustrative and not limiting. It should be understood that modifications and variations can be made by persons skilled in the art in light of the above teachings. Therefore, changes may be made in the particular embodiments disclosed which are within the scope of what is described as defined by the appended claims.

What is claimed is:

1. A method for identifying a low abundance protein in a sample, said method comprising the steps of:

(a) supplying a sample having a low abundance protein physically bound to a high abundance protein;

(b) treating the proteins with a proteolytic agent to generate proteolytic fragments from the proteins;

(c) removing proteolytic fragments of the high abundance protein from the sample; and

(d) identifying the low abundance protein using the proteolytic fragments therefrom.

2. The method of claim 1, wherein step (d) comprises:

characterizing the proteolytic fragments of the low abundance protein; and

identifying the low abundance protein using the characteristics of the proteolytic fragments therefrom.

3. The method of claim 2, wherein the proteolytic fragments of the low abundance protein are characterized using a method selected from the group consisting of chromatograpy, high performance liquid chromatography, electrophoresis, mass spectrometry, and Edman degradation.

4. The method of claim 3, wherein the proteolytic fragments of the low abundance protein are characterized using mass spectrometry.

5. The method of claim 1, wherein the proteolytic agent is a protease or a mixture of proteases.

6. The method of claim 5, wherein the proteolytic agent is trypsin.

7. The method of claim 1, wherein the proteolytic agent is a chemical agent or a mixture of chemical agents.

8. The method of claim 7, wherein the proteolytic agent is cyanogen bromide.

9. The method of claim 1, wherein the proteolytic fragments of the high abundance protein are removed using an immune-absorption method.

10. A method for identifying low abundance proteins in a sample, said method comprising the steps of:

(a) supplying a sample having low abundance proteins physically bound to high abundance proteins;

(b) isolating the high abundance proteins and the low abundant proteins bound thereto from the sample;

(c) treating the isolated proteins with a proteolytic agent to generate proteolytic fragments from the isolated proteins;

(d) removing proteolytic fragments of the high abundance proteins; and

(e) identifying the low abundance proteins using the proteolytic fragments therefrom.

11. The method of claim 10, wherein step (e) comprises:

characterizing the proteolytic fragments of the low abundance proteins; and

identifying the low abundance proteins using the characteristics of the proteolytic fragments therefrom.

12. The method of claim 11, wherein the proteolytic fragments of the low abundance proteins are characterized using a method selected from the group consisting of two-dimentional gel electrophoresis, high performance liquid chromatography, electrophoresis, column chromatograpy, mass spectrometry, and Edman degradation

13. The method of claim 12, wherein the proteolytic fragments of the low abundance proteins are characterized using mass spectrometry.

14. The method of claim 10, wherein the high abundance proteins are isolated using an immune-absorption method.

15. The method of claim 10, further comprising the step of:

identifying low abundance proteins remained in the sample after the isolation of the high abundance proteins.

16. The method of claim 15, wherein the low abundance proteins remained in the sample after the isolation of the high abundance proteins are identified by a method comprising the steps of:

treating said sample after the isolation of the high abundance proteins with a proteolytic agent to generate proteolytic fragments from the low abundance proteins;

characterizing proteolytic fragments of the low abundance proteins; and

identifying the low abundance proteins using the characteristics of the proteolytic fragments of the low abundance proteins.

17. A method for quantifying a low abundance protein in a sample, said method comprising the steps of:

supplying a sample having a low abundance protein physically bound to one or more high abundance proteins;

treating the proteins with a proteolytic agent to generate proteolytic fragments from the proteins;

quantifying proteolytic fragments of the low abundance protein; and

quantifying the low abundance protein based on the quantity of the proteolytic fragments therefrom.

18. The method of claim 17, further comprising the step of:

removing proteolytic fragments of the one or more high abundance proteins from the sample prior to the quantifaction of proteolytic fragments of the low abundance protein.

19. A kit for detecting low abundance proteins in a sample, said kit comprising:

a proteolytic agent capable of fragmenting proteins in the sample and disrupting associations between low abundance proteins and high abundance proteins in the sample; and

a binding material that binds specifically to proteolytic fragments of the high abundance proteins.

20. The kit of claim 19, wherein the binding material is attached to a solid supporting material to facilitate the removal of the binding material with bound proteolytic fragments of the high abundance proteins from the sample.

ABSTRACT

The present invention discloses a novel procedure of removing high abundance proteins (HAPs) from a test sample without the concurrent removal of low abundance proteins (LAPs) that are bound to the HAPs in the test sample. The procedure therefore allows the accurate detection of LAPs without the interference from the HAPs in the test sample. Specifically, the present invention provides methods for detecting LAPs in a test sample by treating the test sample with a proteolytic agent to release HAP-bound LAPs from the HAPs by fragmenting both HAPs and LAPs, removing the HAP fragments from the test sample, analyzing the LAP fragments, and identifying the LAPs in the test sample based on the characteristics of the LAP fragments. The present invention is most useful for the identification of LAPs that are bound to the HAPs in the test sample and are otherwise hard to separate from the HAPs.

Title: METHOD FOR TECTING A LOW ABUNDANCE PROTEIN IN EST SAMPLE
Inventor(s): H. Bryan BENTE
Contact Name: Ronald C. Hudgens (978) 681-2404
Attorney Docket No.: 10021053-1

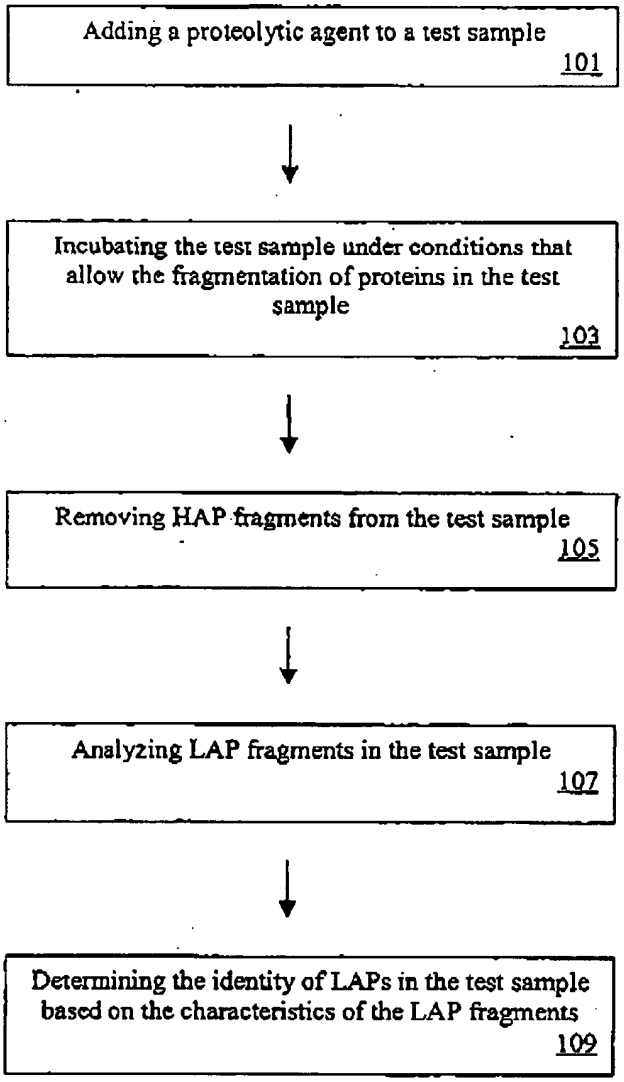

FIG. 1

Title: METHOD FOR ' 'ECTING A LOW ABUNDANCE PROTEIN IN / 'EST SAMPLE
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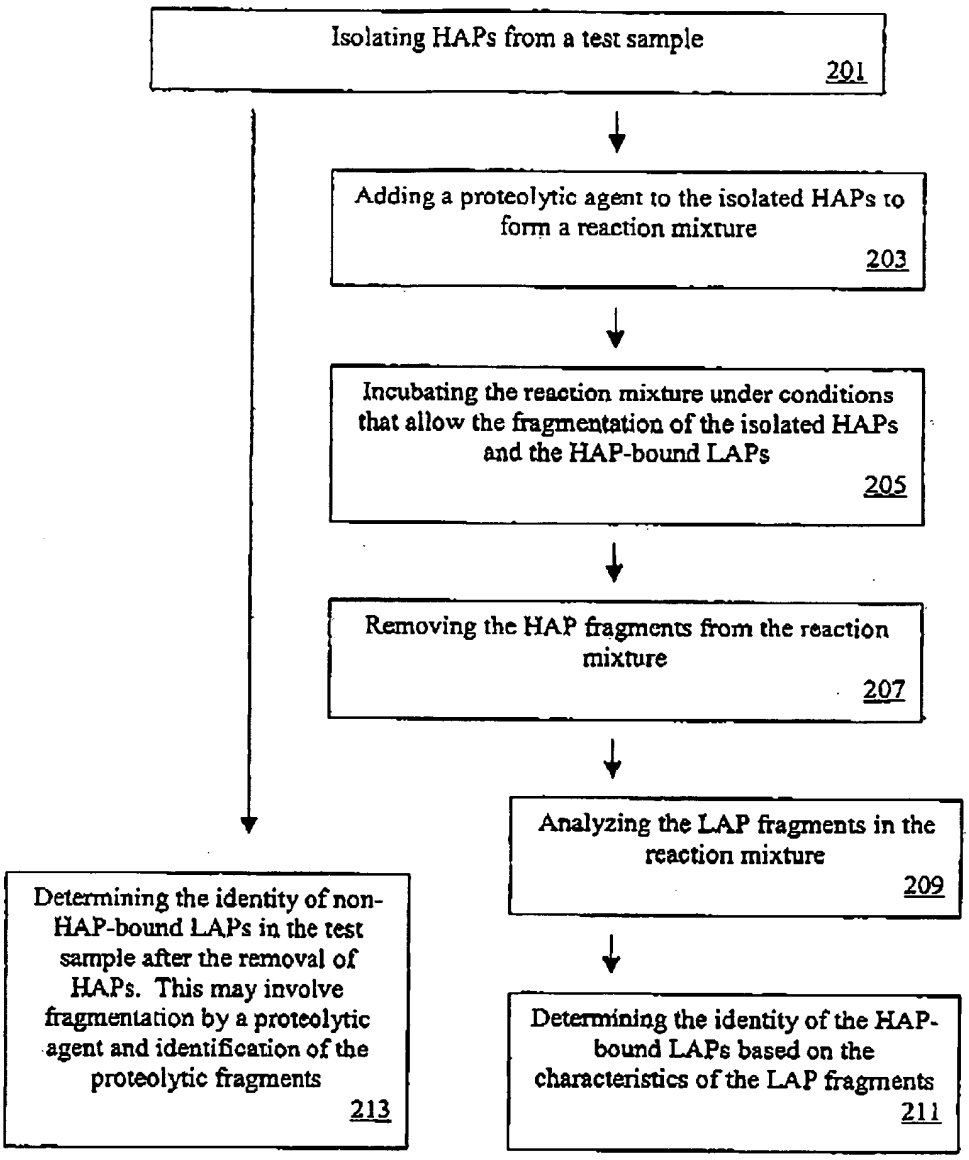

FIG.2

research articles Journal of proteome research

Targeted Proteomics of Low-Level Proteins in Human Plasma by LC/MS^n: Using Human Growth Hormone as a Model System

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This paper describes the profiling of human growth hormone (hGH) in human plasma in order to assess the dynamic range of the ion-trap mass spectrometer for proteomic studies of complex biological samples. Human growth hormone is an example of a low-level plasma protein in vivo, present at sub-femtomole levels. This study was performed on a plasma sample in which hGH has been spiked at 10-fold above the natural level, that is approximately 16 pg/μL of plasma. Initially, the measurement was carried out without any sample enrichment and consisted of the following steps: the full set of plasma proteins were reduced, alkylated, and digested with trypsin, and the resulting peptides were separated on a capillary C-18 column and then detected by ion-trap mass spectrometry (1D LC/MS). In addition, this study provided a global view of the serum proteome with over 200 plasma proteins being preliminarily identified. In the MS/MS analysis, hGH was detected by characterization of the first tryptic peptide (T_1). The initial identification was confirmed by alternative approaches, which also allowed the evaluation of different sample purification protocols. First, the plasma sample containing hGH was fractionated on a reversed-phase HPLC column and digested, and hGH could now be identified by MS/MS measurements of two tryptic peptides (T_1 and T_4) by the same 1D LC/MS protocol. In addition, the assignment of peptide identity was made with higher certainty (as measured by an algorithm score). The plasma sample was also fractionated by 1D and 2D gel electrophoresis, the selected bands were digested and analyzed again by the 1D LC/MS protocol. In both cases using the gel prepurifications, hGH was identified with additional peptides. Finally, the plasma sample was analyzed by 2D chromatography (ion exchange and reversed phase) on a new instrumental platform (ProteomeX), and hGH was identified by the observation of five tryptic peptides. In conclusion, these experiments were able to detect growth hormone in the low femtomole level with a dynamic range of 1 in 40 000 by several independent approaches. The amount of growth hormone, while 10-fold above normal *in vivo* levels, represents concentrations that may be present in disease states (such as acromegaly) and also in doping control measurements. These studies have demonstrated that shotgun sequencing approaches (LC/MS/MS) not only can profile high-abundance proteins in complex biological fluids but also have the potential to identify and quantitate low-level proteins present in such complex mixtures without extensive prepurification protocols. A key to such studies, however, is to use targeted approaches that reduce the complexity of the solute mixture that is presented to the mass spectrometer at a given time point. The various sample preparation protocols described here all improved the quality of the hGH measurement, although in this study the 2D chromatographic approach gave the greatest sequence coverage.

Keywords: 2D LC/MS • shotgun sequencing • proteomics • clinical application • human growth hormone • ion-trap mass spectrometry

Introduction

The study of the plasma proteome is a very challenging exercise in that the sample contains a large number of different proteins, together with proteins that contain a diversity of post-translational modifications, especially glycosylation and phosphorylation. Furthermore, plasma proteins are present over a wide dynamic range from very high levels of albumin to low levels of hormones, regulatory and other proteins. One approach to proteomic studies is termed shotgun sequencing, which may, or may not, be used in combination with 1 or 2D gel technologies.[1-3] In the shotgun sequencing approach, the components of a proteomic sample are digested with a suitable protease and the resulting peptides are then resolved by chromatography and detected by mass spectrometry (LC/MS/MS). The chromatographic process consists of one or more steps that are based on different physical properties of the

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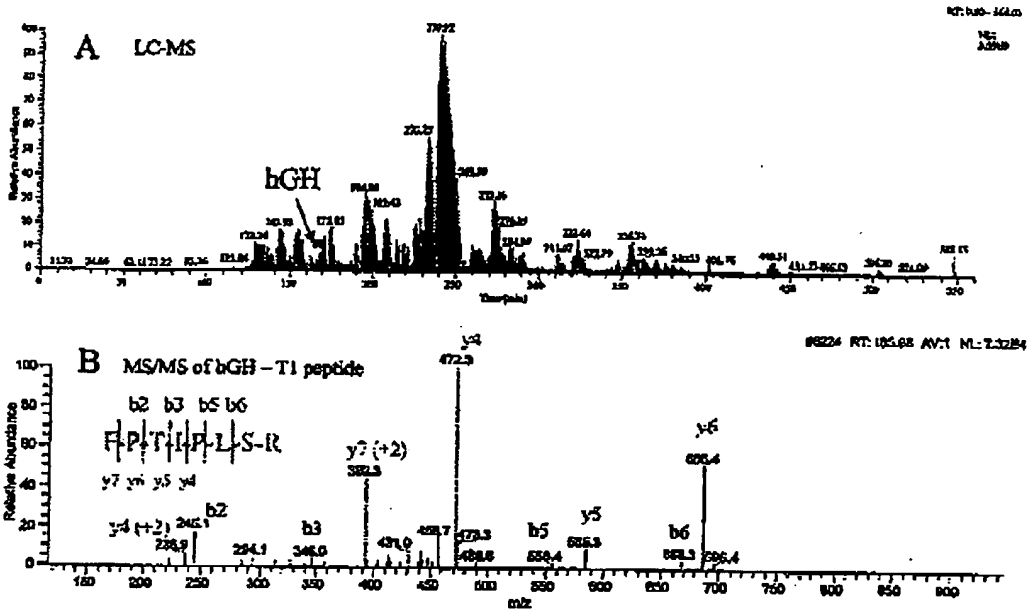

Figure 1. Tryptic map of the peptides obtained from a mixture of reduced plasma proteins with an add back of low levels of human growth hormone. The tryptic digest of the mixtures of 16 fmol of human growth hormone (hGH) and 640 pmol of plasma proteins was loaded onto a capillary C-18 LC column for LC–MS analysis (see the Experimental Section for details). (A) Resulting elution profile. In the tryptic map, the MS/MS scan at 166 min was matched against a human database and assigned to the T1 peptide corresponding to hGH. (B) MS/MS fragmentation of the T1 peptide with labeling of the major fragmentation sites.

peptides, such as a combination of ion-exchange chromatography and reversed-phase HPLC.[1]

In this study, a variety of different protocols C-4 RPLC 1 and 2D gel electrophoresis were explored to prefractionate the plasma sample containing hGH before trypsin digestion and separation by 1D reversed-phase HPLC–MS analysis. The results were compared with corresponding results from direct 1D reversed-phase HPLC–MS analysis. In addition, a 2D approach (combination of ion exchange and RPLC) was performed on a new instrumental platform, ProteomeX. While direct analysis of a digest has the benefit of simplicity, the use of both gels (1D and 2D) and the 2D HPLC approach gave a greater degree of sequence coverage of hGH. With sample prefractionation, the mass spectrometer is not required to measure such a high degree of heterogeneity per unit of time and a greater number of components will be identified.

The dynamic range of the shotgun sequencing approach using ion-trap mass spectrometry was explored by not removing high-level plasma proteins from the plasma sample before trypsin digestion. In addition, the results could be related to the concentration of a low-level plasma protein, namely hGH.

Methods

Sample Preparation. A plasma sample (lyophilized, Sigma, 20 μg/μL) and hGH (0.6 ng/μL) were reduced by DTT (dithiothreitol, Sigma) and alkylated with iodoacetic acid (1 M in NaOH solution, Sigma). As shown in Figure 1, 1000 μL of sample was loaded onto a column (Vydac C-4, 2.1 mm i.d. × 15 cm) and eluted from the column (in approximately 2 mL of TFA, trifluoroacetic acid/acetonitrile solvent), and the sample was dried down and reconstituted with trypsin–digestion buffer (in 1 mL of 0.1 M ammonium bicarbonate solution). The fractions from the RPLC chromatography were treated with trypsin (1:50 w/w) at room temperature for 12 h. To ensure complete digestion, another aliquot of trypsin (Promega, concentration of 1 μg/ μL, 1:50, w/w) was added, and the digestion was continued for a total of 24 h.

In each of the sample preparation studies, the amount/concentration of growth hormone used in the experiment was adjusted to allow for experimental variables such as degree of dilution, injection volume, etc. so that the amount of hGH analyzed was consistent with the concentration described in the abstract, namely 16 pg/μL of plasma.

HPLC and Mass Spectrometry Measurements. The HPLC separation was performed on a Surveyor LC system (Thermo-Finnigan, San Jose, CA). The flow rate was maintained at 150 μL/min before splitting and at 1.5 μL/min after the flow split. The gradient was started at 2% AcCN dissolved in 0.1% formic acid for 3 min, ramped to 60% AcCN in 180 min, and finally ramped to 80% AcCN for another 20 min. An aliquot (5 μL) of the sample solution (in a 10 μL sample loop) was injected from the autosampler (using the no-waste mode) onto a C-18 capillary column (Biobasic C-18, Thermo Keystone-Hypersil, 180 μm × 10 cm), which was connected to an ion source chamber (orthogonal) with a sheath gas flow at 3 units for MS analysis. The temperature of the ion transfer tube was set at 140 °C. The spray voltage was set at 2.8 kV, and the normalized collision energies were set at 35% for MS/MS. Dynamic exclusion was used at an exclusion duration for 5 min. Data-dependent ion selection was set to trap the interesting ions (e.g., hGH tryptic fragment ions) from the previous MS/MS scan.

Table 1. Identification of Proteins Present in Trypdc Digest of a Human Plasma Digest Containing an Add-Back of Growth Hormone[a]

protein i.d. score[b]	protein description[c]	no. of scans[d]	score[e]	normalized score[f]	unique peptides[g]
1	human serum albumin	35	136534	3901	17
2	fibrinogen, α chain	18	106686	5927	11
3	α-2 macroglobulin	12	64671	5389	9
4	fibrinogen-δ-chain	8	55094	6887	4
5	fibrinogen-β-chain	8	49547	6193	5
6	apolipoprotein A−I	11	42787	3890	7
7	transferrin	11	42787	3478	7
8	haptoglobulin related protein	12	41736	4229	6
9	human complement C3	8	21843	2730	5
10	apolipoprotein C−I	1	18058	2058	1
221	protein phosphatase 2A, B56-γ I	1	882	882	1
222	protein kinase (EC 2.7.1.37) cdc2-related protein	1	880	880	1
223	putative glialblastoma cell differentiation-related protein	1	879	879	1
224	JTV1: hypothetical protein PRO0992	1	878	878	1
225	glutamate receptor, metabotropic 5	1	873	873	1
226	AF302154_1 SPG protein	1	871	871	1
227	transferrin binding protein B; TbpB (*Moraxella catarrhalis*)	1	868	868	1
228	human growth hormone	1	863	863	1
229	DNA-binding protein pAT 133	1	862	862	1
230	hypothetical protein FLJ20354	1	860	860	1

[a] The LC/MS/MS analysis was performed as in the Experimental Section. [b] The protein i.d. is related to the probability of the assignment. [c] The protein description is based on assignment from TurboSequest. The nomenclature has been simplified to reflect the i.d. of the parent protein. [d] The no. of scans column gives the number of peptides (either the same or different peptides) that are found in the protein. [e] The score is the summation of the three match factors from the database search result (Sp, Xcorr, and δCn). [f] The normalized score is the score divided by the number of scans. [g] The unique peptides column gives the number of peptides with different sequences that are assigned to the same protein.

The 2D separations were performed on a ProteomeX system, which comprises an auto sampler, two HPLC pumps, a 10-port column-switching valve, and a Deca-XP ion-trap mass spectrometer with a micro-electrospray interface. The 10-port valve allows loading of a subsequent ion-exchange fraction onto the second reversed-phase column while the first one is performing LC-MS/MS analysis. The micro electrospray interface comprises of a 30 μm metal needle that is orthogonal to the inlet of the ion-trap mass spectrometer. For the capillary separations, a flow rate of 1−2 μL/min was used. In the first step, a strong cation exchanger (BioBasic SCX, 0.32 mm × 10 cm, Thermo Keystone/Hypersil, Allentown, PA) was used, and then a reversed-phase (BioBasic C18, 300 Å, 5 μm silica, 180 μm × 10 cm, Thermo Keystone/Hypersil, Allentown, PA) capillary column was used for the second dimension.

Bioinformatics. The sequences of the uninterpreted CID spectra were identified by correlation with the peptide sequences present in the nonredundant protein sequence database (OWL Version 30.3) using the SEQUEST algorithm (Version C1) incorporated into the ThermoFinnigan BIOWORKS software (version 3.0).[5] The SEQUEST search results were initially assessed by examination of the Xcorr (cross correlation) and ΔCn (delta normalized correlation) scores. The Xcorr function measures the similarity between the mass-to-charge ratios (*m/z*) for the fragment ions predicted from amino acid sequences obtained from the database and the fragment ions observed in the MS/MS spectrum. The ΔCn score is obtained by normalizing the Xcorr values to 1.0 and observing the difference between the first- and second-ranked amino acid sequences.[5] Thus, the ΔCn score discriminates between high quality and noisy spectra although both may match a theoretical spectra. As a general rule, an Xcorr value of greater than 2.0 for a doubly charged ion (> 1.5 for singly charged ion) and ΔCn greater than 0.1 was accepted as a positive identification.[5] Manual inspection key spectra were performed to confirm the SEQUEST result. Bioworks is a new version of TurboSequest in which the three matching factors (Sp, Xcorr, and δCn) are used to construct a unified ranking score[5] (see Table 1). A higher-ranking score is generally associated with a greater probability for a correct assignment of a particular protein sequence.

1D and 2D Gel Electrophoresis. A sample aliquot (5 μL) containing hGH (15 ng/μL) and human plasma (20 μg/μL) was loaded on a 14% mini-Laemmi gel and stained with Coomassie dye (R350, Amersham) after electrophoresis. The gel bands indicated by the boxes in Figure 3 were cut out, reduced, alkylated, and digested with trypsin as described above, and the resulting peptides were dissolved in buffer A (100 μL) for LC/MS analysis.

In a 2D gel analysis, the plasma sample (or hGH control) was separated on a 18 cm, pH 3−10 IPG strip (linear strip, Amersham) for the first dimension and then on an 18 × 18 cm, 14% cross-linked PAGE (polyacrylamide gel electrophoresis) gel as the second dimension. The sample solution contained a mixture of human plasma proteins (20 μg/μL) and hGH (12 ng/μL), and the standard solution contained human growth hormone only (0.1 μg/μL). As shown in the Figure 3, 80 μL of each was loaded onto the gel. Protein spots were cut from the gel, reduced by DTT, alkylated by iodoacetamide, and digested with trypsin (see below), and the resulting peptides were recovered into 100 μL of solution.

In-Gel Digestion. Protein spots/bands (1−3 mm per spot/band) were cut from the gels, placed into individual prewashed microtubes with 100 μL of 50% acetonitrile/0.2 M ammonium carbonate (pH 8), and incubated for 30 min at room temperature. The procedure was repeated two more times to remove SDS and stain. The gel plugs were broken into several smaller pieces using a scalpel. The gel pieces were then dehydrated by first adding 30−50 μL of 100% acetonitrile, just enough to cover all gel pieces. After 10 min, the excess acetonitrile was removed and the gel pieces taken to dryness on a SpeedVac over 45−60

min. The dried gel pieces were rehydrated with a concentrated trypsin solution (20 μg/mL) using a volume that was equivalent to the volume of the original gel plug from whence they came. Rehydration was allowed to proceed for 20−30 min. At that point, just enough 50 mM NH_4HCO_3 (pH 8) buffer was added to completely cover the gel pieces, and the mixture was allowed to incubate for 12−20 h at 37 °C. This incubation supernatant was removed and saved. Then 10% formic acid was added, just enough to cover the plug, and the mixture was incubated at 60 °C for 10 min and then sonicated for 1 h. This formic acid supernatant was combined with the incubation supernatant (to approximate a total of 100 μL solution).

Results and Discussion

Figure 1A shows the LC/MS profile for a human plasma sample that was reduced and digested with trypsin, and the resulting peptides were separated by an 8 h reversed-phase HPLC (RPLC) gradient. The analysis was performed on a sample that contained an add-back of human growth hormone (which had been reduced and trypsin digested in the same manner as the plasma sample). As was described previously,[7] the LC/MS analysis showed good reproducibility in terms of protein identification, with the variability being concentrated in the low-level proteins. It is clear from the elution profile and subsequent MS measurement that some peptides are present in very large amounts, which are generated from the high-abundance plasma proteins such as albumin. A concern is that such major peptides will interfere with the detection of minor peptides, particularly as HPLC separations typically exhibit peaks with some degree of fronting and tailing.[8] Thus, the presence of growth hormone in known, low levels could allow determination of the dynamic range of this measurement and assess the importance of sample preparation.

The approach to proteomic analysis described in this paper relies on programmed MS/MS fragmentation performed on selected ions generated from the peptide mixture present at a given time point in the RPLC separation. The resulting MS/MS data sets were then matched with predicted spectra from genomic or proteomic databases.[1] Typically, the ions for MS/MS analysis are selected on the basis of signal intensity, although other selection criteria are possible, e.g., neutral loss. The software automatically assigns MS/MS spectra to peptide sequences, measures a probability for this assignment, and then matches peptides to possible protein identifications (ID).[1,9] In the analysis of such a complex mixture, a substantial number of peptides (usually 15 or more components) were present in each MS analysis over a given time slice (30 s) of a typical chromatographic peak (1−2 min). Three MS/MS scans were programmed between each MS measurement to improve the ability of the mass spectrometer to detect low-level peptides. In addition, the use of a new version of the ion-trap mass spectrometer (DecaXP) facilitated the characterization of low-level components due to hardware modifications in the MS system, which resulted in improved sensitivity.[10]

In this manner, a single peptide was found in the plasma sample for human growth hormone, which corresponded to the first tryptic or N-terminal peptide (T1). In Figure 1, the lower profile (B) shows the MS/MS spectra of this peptide with the resulting b and y ion fragments. Although a single peptide is being used for the protein ID, the quality of MS/MS spectra (b and y ions, see Figure 1), facilitated by strong fragmentation on the N-terminal side a proline residue,[10] was sufficient to give a preliminary identification of human growth hormone. The amount of growth hormone detected in this analysis represented a dynamic range of 1 in 40 000 and corresponded to the levels of growth hormone that have been observed in disease states such as acromegaly.[11] The circulating level of growth hormone is 10-fold lower in normal patients, and future studies will be directed at improving the sensitivity of the analysis to allow measurement of normal levels of this protein.

Table 1 shows a selected portion of the results from proteomic analysis of the plasma sample. It can be noted that the 10 most abundant proteins correspond to generally recognized major components of plasma, e.g., albumin, fibrinogen, macroglobulin, apolipoprotein A-1 and C-1, and transferrin. In addition, most of the high-level proteins were present in many MS scans indicating high abundance. In the case of albumin, the protein was identified with high sequence coverage (17 unique peptides) and fibrinogen, macroglobulin, with 11 and 9 peptides, respectively. These results demonstrate that, even with a complex biological sample, it is possible to get relatively high sequence coverage of proteins provided they are present at significant levels. The proteins that are present in much lower amounts are represented by the lower list in Table 1, labeled proteins 221−230. The proteins are ranked by a score that is the summation of three match factors (Sp, Xcorr, and ΔCn) and the probability measurements of these factors (see the Experimental Section). It can be noted that each of the assignments of these low-level proteins is achieved with only one scan and one unique peptide, and thus, such assignments must be considered to be preliminary until confirmed by other analytical approaches. Although the MS/MS data clearly identify the T1 peptide of human growth hormone, only one MS/MS scan was obtained that specifically identifies hGH in this highly complex plasma mixture. For this reason, a poor score (863) and low ranking order (number 228) were obtained for hGH in this chart, reflecting the relatively low abundance of hGH in this complex protein mixture. This assignment was strengthened by the observation of other growth hormone peptides by a RPLC prefractionation step (see below).

A secondary measurement on the plasma sample containing hGH was performed using sample enrichment on a C4 reversed-phase HPLC column. As shown in Figure 2, the majority of small molecules (e.g., urea, dithiothreitol) as well as the less hydrophobic proteins eluted from the column in the early part of the elution profile at an isocratic step with an organic modifier concentration of 10% acetonitrile. A gradient of acetonitrile was then performed to elute the more hydrophobic proteins. Growth hormone is known to be a relatively hydrophobic protein and thus well retained on a reversed-phase HPLC column,[12] which then allowed for RPLC to be used as an effective sample enrichment step. This is an example of targeted proteomics where the sample purification protocol can be optimized to a single protein (or group of proteins).

Three pools were prepared, from fractions 1−3, 4−6, and 7−9, concentrated, and digested with trypsin and then analyzed by the 1D LC/MS protocol. The target analyte (hGH) was characterized in fractions 4−6 but was not detected in the other two fractions (data not shown, see Figure 2). With the enrichment step, hGH was now detected with greater certainty as both T1 as well the T4 peptide were characterized by MS/MS analysis. In addition, the peptide T4 had considerable MS/MS fragmentation information that further improved the quality of the ID from databases. In summary, more scans (the number of peptides) and a higher score (25235) and the ranking order (number 13) show the value of RPLC pre-fractionation. An

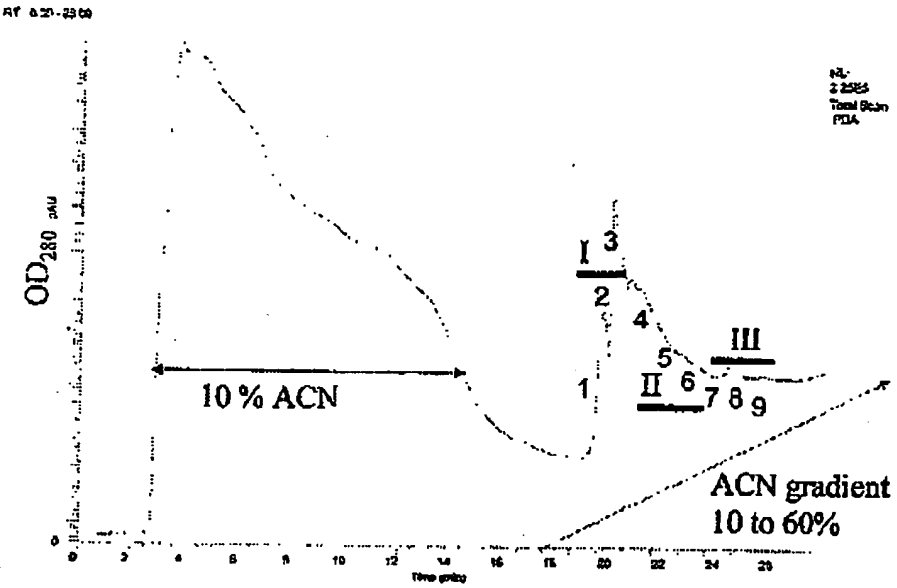

Figure 2. Enrichment of hGH from a trypsin digest of human plasma proteins via C-4 RPLC column (2.1 mm i.d. × 15 cm). A 1 mL aliquot of reduced and alkylated human plasma proteins (20 μg/μL) with an add-back of human growth hormone (~0.6 ng/μL) was loaded onto a C-4 reversed-phase column. The column was eluted as follows: buffer A, 0.1% TFA in water; buffer B, 0.1% TFA in acetonitrile; isocratic at 10% B for 15 min and then a gradient to 60% B in 20 min. The fractions from the acetonitrile gradient were pooled and dried down as three aliquots (fraction I (1–3), II (4–6), and III (7–9)) and then reconstituted with trypsin-digestion buffer (approximately 1 mL) and digested as described in the Experimental Section.

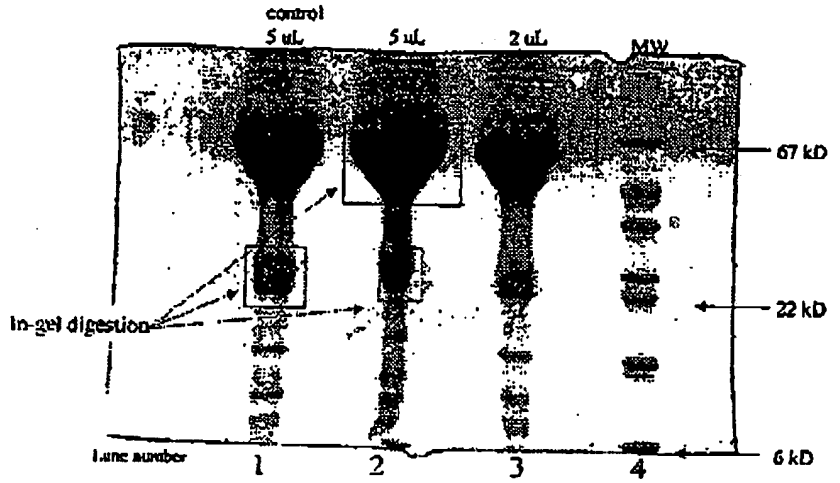

Figure 3. Sample pre-fractionation on a 1D SDS–PAGE gel. The conditions are described in the Experimental Section. The bands taken for in-gel digestion are shown by boxes, and the left-hand side of the gel contains the MW markers that allowed calibration of the gel. The MW of relevant markers are listed. Lane 1 contains a load of 5 μL of the control (plasma with no add-back of hGH), lane 2 contains a 5 and 2 μL loading, respectively, of the plasma sample containing the hGH add-back, while lane 4 contains the MW standards.

advantage of such a reversed-phase step is that it is rather generic and can be applied to the enrichment of any protein that can be recovered in good yield. It should be noted, however, that albumin fragments were not completely removed due to the altered chromatographic properties of the fragments relative to the intact protein (which was removed). One could assume that other sample purification protocols would also suffer from similar problems, due to the presence of fragments with altered binding properties relative to the intact molecule.

A popular approach to sample fractionation is to use 1D PAGE electrophoresis of a biological sample and then perform 1D LC/MS analysis on enzyme digests of selected bands. Figure 3 shows the PAGE fractionation of the hGH-containing plasma sample, and in-gel digestion was performed on a band with a

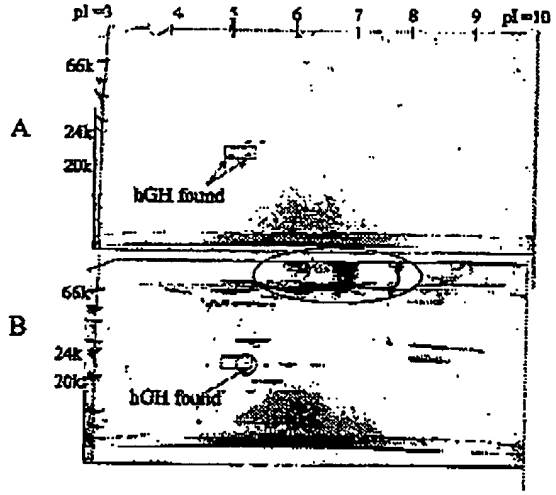

Figure 4. Sample pre-fractionation on a 2D SDS–PAGE/IEF gel. The conditions are described in the Experimental Section. The bands taken for in-gel digestion are shown by boxes. (A) Separation of hGH and isoforms (8 μg loading). (B) Separation of plasma proteins with an add-back of hGH (1600 μg). The large sample caused some distortion of the high-abundance proteins. The images of the gels were shrunk in the vertical direction to reduce the space requirements, and the region above 66 kD in the plasma sample shows high-abundance proteins such as albumin.

MW corresponding to hGH (22 kD). Figure 3 also shows that the large amount of albumin in the sample was effectively removed in a large band at approximately 67 kD. Growth hormone was identified with an improved score and rank, number 44, relative to the unfractionated sample, as well as the characterization of peptide T10 by LC/MS. Although the gel separation was able to achieve a substantial clean-up of unrelated proteins, the complexity of the sample meant that there are still some major proteins that have co-purified with hGH. The contaminants included presumed fragments of major serum proteins (albumin, apolipoprotein A-1, complement factors, various immunoglobulins) as well as adventitious contaminants such as trypsin and keratins.

Figure 4 shows the fractionation of a sample of hGH (part A) and the plasma sample containing hGH on a 2D gel (section B). The control sample showed more than one band due to degradative reactions such as deamidation and proteolysis that had occurred on sample storage.[18] After fractionation of the plasma sample by isoelectric focusing (IEF) and SDS–PAGE, the area corresponding to the hGH control was digested and analyzed by 1D LC/MS/MS. The purpose of this study was to compare different sample preparation protocols with 1- or 2D HPLC separations, and thus, direct infusion of a sample into the MS (as in static nanospray) was not performed. It can be noted, however, that while static nanospray can give improved sensitivity, this approach is less suited to complex mixtures. In this analysis, hGH was identified with an improved score and ranking (number 4) and by the characterization of two peptides, T2 (position 8–16) and T4 (position 20–38) peptides (data not shown). Apart from the usual contaminants, the LC/MS/MS analysis was much less complex than the analysis of other sample preparation steps as the Sequest program made only approximately seven other probable protein assignments.

The results from the analysis of the plasma sample containing hGH with the 2D separation provided by the ProteomeX are shown in Figure 5. In this analysis, the sample was separated into five isocratic salt steps (flow through, 50, 100, 200, and 500 mM ammonium chloride) on a strong cation ion-exchange column (SCX). Each fraction was then loaded on a

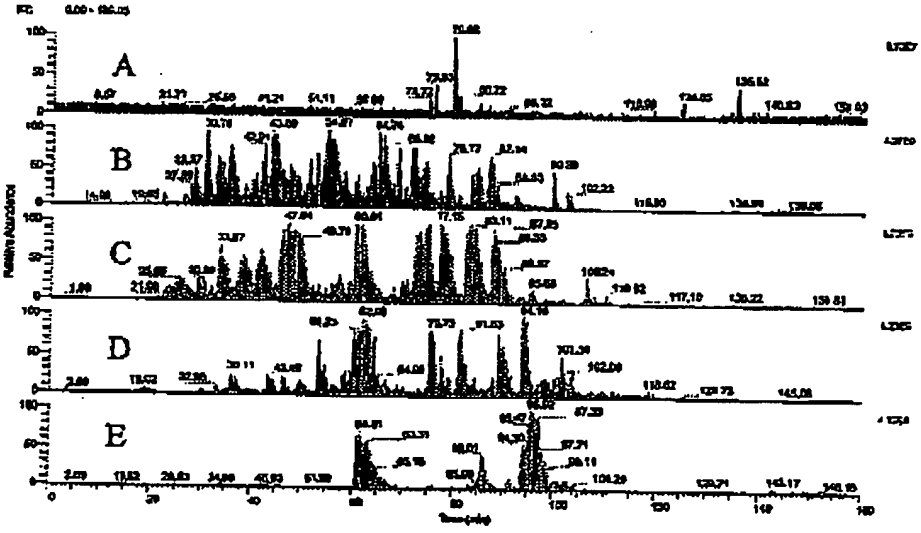

Figure 5. Fractionation of a trypsin-digest of plasma containing an hGH add-back using 2-dimensional chromatography. The conditions for the 2D separation (IEX and RPLC) are described in the Experimental Section. (A, B, C, D, and E) Elution profiles for the separation of the peptides derived from plasma proteins contained in a given ion-exchange fraction (from an isocratic elution step of 0, 50, 100, 200, and 500 mM ammonium chloride, respectively). The number of peptide based protein identifications for each of the steps are as follows: 7, 134, 98, 68, and 26.

C_{18} reversed-phase column and separated by a linear gradient of acetonitrile (see the Experimental Section). Growth hormone was identified by five different peptides (T2, 8,11, 15, and 18–19) as well as the preliminary identification of over 200 other plasma proteins (data not shown). The improved sequence coverage of the target protein hGH can be attributed to the better resolving power of the 2D chromatographic process, so that the mass spectrometer has a greater opportunity to sequence low level peptides in the MS/MS analysis. Thus, one can see that measurement of the dynamic range of an ion trap in such a complex analysis is partly dependent on the degree of preseparation of the peptide mixture. The degree of sequence coverage of the target protein could be improved by optimization of the 2D separation by either predictive studies or calibration of the separation with a digest of the target protein. In the separation shown in Figure 5, it can be seen that all of the identified peptides elute in just three of the five ion-exchange fractions. When compared with the gel preparative steps, the 2D HPLC method has the advantage of less sample manipulation, absence of gel artifacts, and losses on extraction.[16]

In conclusion, these studies demonstrate that shotgun sequencing with LC/MS/MS is able to detect low-level plasma proteins despite the complexity of the sample. Although the presence of high-level proteins does reduce the ability to detect very minor components, such an analytical approach does offer simplicity and speed of analysis. This approach can also be viewed as showing some aspects of high throughput proteomic analysis in that more than 200 proteins were identified in an 8-h period with minimal sample preparation. This contrasts with the use of 1- or 2D gels where a lengthy time is required to run the gel, cut out the bands or spots, and perform subsequent MS analysis and/or in approaches that rely on extensive immunoaffinity depletion steps. It should be noted that the 1D reversed-phase HPLC step was used in all analyses (except for 2D HPLC) so as to allow comparison of the efficiency of the different sample purification protocols. Also the shotgun sequencing approach should be of use in the study of very low level samples, which are not readily characterized in-gel studies.[15] However, we believe the two approaches are complementary in that the gel offers protein patterns, allows an archive of the sample, and provides additional quantitative information as well as a visual record of the sample.[18]

This proteomic study can also serve as an example of targeted proteomics in which LC/MS/MS is directed at a specific protein or pathway. For example, Simpson et al.[4] used this approach to detect a low-level tumor marker. Such studies can be facilitated by the identification of a specific or diagnostic peptide,[17] e.g., the N-terminal peptide of hGH and the exploitation of its chromatographic and mass spectrometric properties. Previous studies have demonstrated that such targeting (mass selection) with selected ion monitoring (SIM) can allow the characterization of a low level protein that is otherwise undetectable.[4] Furthermore, we believe that such studies will have application in the development of novel therapeutics polypeptides via mechanistic or in metabolic studies.

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